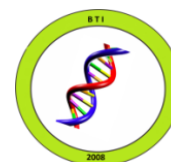




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Research Article

CELL MEDIATED IMMUNE RESPONSE AGAINST FRACTIONATED LIPOPOLYSACCHARIDE (LPS) ANTIGENS OF *BRUCELLA MELITENSIS* 16M IN CELL CULTURE

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ABSTRACT

Cell mediated immunity is an important part of host defence. Nitric oxide has an antimicrobial activity and play an important role against variety of bacteria including *B. melitensis*. This study aimed to evaluate the immunoreactivity of fractionated lipopolysaccharide (LPS) antigen of *B. melitensis* in cell culture. The LPS antigen was fractionated by gel filtration chromatography. The lymphoproliferation was estimated with 3-(4,5-dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT) colorimetric method. After comparing all the LPS antigen fractions which were fractionated by gel filtration chromatography, it was observed that LPG1 induced maximum lymphocyte proliferation *in-vitro*, followed by LPG2, LPG5, LPG4 and then LPG3 respectively. Nitric oxide (NO) production by the monocyte-derived macrophages on stimulation with LPS antigen of *B. melitensis* was assessed using a colorimetric assay for nitrite based on Griess reaction. It was observed that LPG1 produce maximum nitrite followed by LPG2, LPG3, LPG5 and LPG4 respectively. It was observed in this study that LPG-1 and LPG-2 fractions of lipopolysaccharide antigens induced maximum proliferation of lymphocytes and also induces monocytes to produce significant amount of NO. These fractionated LPS were found immunoreactive and will further be tested for the development of immunodiagnostic kit, which will be useful to detect the presence of *anti-Brucella* antibody in the infected host.

Keywords: *B. melitensis*, Nitric oxide, Lymphocyte transformation test, Gel filtration chromatography, cell mediated immunity, LPS antigen, monocytes

INTRODUCTION

Brucellosis is a most common bacterial zoonosis throughout the world. It was first identified in 1860s by David Bruce in Malta and became popularly known as Malta fever (Pappaset *al.*, 2006;

Araj, 2010). This causative agent of this diseases is *Brucella*, which used domestic and wild animals as its natural reservoirs (Pappaset *al.*, 2005). It is endemic in many areas of the world, characterized by chronic infections in animals leading to

abortion and infertility, and a systemic, febrile illness in humans (Paulsen *et al.*, 2002). *Brucella* can escape the killing mechanism within the macrophages therefore able to establish chronic infection in the concerned host (Baldwin and Winter, 1994; Sangari and Aguero, 1996). *Brucella* is facultative intracellular pathogens that survive and replicate in both phagocytic and non-phagocytic cells (Dubray, 1985).

The host immune response can be functionally classified into innate and adaptive immunity. The innate immune response is the first line of defence against invading pathogens. Its elements include physical barriers (skin and internal epithelial layers), humoral components (various chemokines, complement system and opsonins) and cellular components such as phagocytes (neutrophils, monocytes, macrophages and dendritic cells) and innate lymphocyte subsets (natural killer cells and $\gamma\delta$ T cells) (Golding *et al.*, 2001; Jiang *et al.*, 1993; Kubota K, 2010; Parkin and Cohen 2001). Adaptive immunity can be classified into cell-mediated immunity and humoral immunity. T lymphocytes play a major role in cell-mediated immunity via cytokine production and by exerting cytotoxic effects. On the other hand, antibody-producing B lymphocytes are the major cells in adaptive humoral immunity (Parkin and Cohen 2001).

Cell mediated immunity (CMI) plays an important role in protection against intracellular pathogen such as *Brucella* spp. It is characterized by antigen-specific T cell mediated activation of macrophages, which are the major effectors of cell-mediated killing of *Brucella* (Nicoletti and Winter, 1990;

Oliveira, 1998). T lymphocyte plays an important role in cell mediated immunity via cytokine production and by exerting cytokine effect. On the other side, antibody producing B lymphocytes are major cells in adaptive humoral immunity (Parkin and Cohen, 2001). *Brucella* spp. Have the ability to survive and replicate within host cells especially macrophages. Protection of host against *Brucella* is dependent on activated antigen-presenting cells (APCs) in innate immunity and on activated T_H cells and T_C cells in adaptive immunity to remove the organisms and infected cells (Baldwin and Goenka 2006; Skendros *et al.*, 2011).

Nitric oxide (NO) has potent antimicrobial activity and also plays an important role to kill some bacteria. It can also combine with the superoxide anion to yield potential antimicrobial substances. Recent evidence suggests that much of the antimicrobial activity of macrophages against bacterial, fungal, helminthic and protozoal pathogens is due to nitric oxide and substances derived from it (Verma *et al.*, 2016). *In-vivo* macrophages, neutrophils, endothelial cells, keratinocytes and fibroblasts lead to the production of NO. It also serves as an intercellular messenger and toxic defense molecule for nonspecific immune responses to infectious pathogens (Sasaki *et al.*, 1998). NO-related antimicrobial activity has been demonstrated *in vitro* against a broad range of pathogenic microorganisms, including 3 viruses, bacteria, fungi, and parasites (Groote and Fang, 1995).

NO also involved in host defences against intracellular pathogens and might play a role in persistent or latent infections (Fang, 1997). NO is derived from L-

arginine in a reaction catalysed by the enzyme NO synthase (NOS), of which three different isoforms have been identified (Nathan and Xie, 1994). The inducible isoform of NOS (iNOS) is responsible for the high output path of NO production involved in antimicrobial activity (Nathan, 1997). iNOS expression is induced by proinflammatory cytokines such as gamma interferon (IFN- γ), tumor necrosis factor, alpha, and interleukin 1 (IL-1), as well as by microbial products such as LPS and lipoteichoic acid (Fang, 1997). In the present study, an attempt had been made to study the lymphocyte proliferation and production of NO by monocyte-derived macrophages in response to different LPS antigens of *B. melitensis* 16M in cell culture.

MATERIAL AND METHODS

Experimental animal

Animals used in this study were provided by Animal Health Division, CIRG, Makhdoom, India. Total of 6 goat used in this study, 5 goat injected subcutaneously with fractionated LPS antigen of *B. melitensis* 16M, while the rest 1 goat was injected with PBS and served as control.

Preparation of Lipopolysaccharide (LPS) Antigens

Previously described method was used for the preparation of LPS antigens (Ugalde et. al., 2000). Cells were suspended in 5% acetic acid – 10% NaCl, autoclaved for 30 min. and centrifuged at 10,000 rpm for 15 min. Cell debris were removed and supernatant was precipitated with 5 volume of methanol (1% methanol saturated with sodium acetate), incubated at 4°C for 2-4 hrs. Precipitate were digested with lysozyme, nucleases and proteinase K and extracted it with hot

phenol. After chilled the mixture phenol phase were precipitated with methanol and re-suspended in water, centrifuged at 10,000 x g for 18 hrs at 4°C in an ultracentrifuge. After centrifugation supernatant fluid were chromatographed on sephadex G-50 and void volume fractions were freeze dried.

Fractionation of LPS antigen by Gel filtration chromatography

Antigen fractionation for *B. melitensis* LPS antigen was done by gel filtration chromatography on a 1.5 X 80 cm column bed of Sephacryl S-200 having a total volume (V_t) of 136 ml. Void volume (V_0) of the column bed was 45 ml. The column was pre-calibrated with standard protein molecular weight marker viz. bovine serum albumin (MW 66 KDa) and cytochrome C (MW 12.5 KDa). 2 ml of concentrated LPS antigen containing 80 mg total protein was eluted at the rate of 16 ml per hour with 30 mM Tris buffer (pH 7.5) containing 0.1 M sodium chloride. Absorbance of eluates was monitored at 280 nm. Fractions thus obtained in each region were pooled and were dialyzed against distilled water at 4°C. All the pooled fractions were concentrated by vacuum concentrator and filter sterilized through a membrane filter (0.22 μ m), aliquoted and stored at -20°C for further use. Each fraction LPS antigen which was eluted in gel filtration chromatography were further named as LPG-1, LPG-2, LPG-3, LPG-4 and LPG-5 respectively.

Lymphocyte Transformation Test

Lymphocyte Transformation Test in response to fractionated LPS antigens was performed as per the previously described method (Gupta et al., 2004). Blood was collected separately from

external jugular vein of each goat into sterilized assembly containing 2.7% EDTA in ratio 20:1. Peripheral blood mononuclear cells (PBMNCs) were isolated from 10 ml of venous blood by centrifugation at 2000 rpm on histopaque at room temperature for 30 min. PBMNCs were removed from the interphase and were washed three times in sterilized PBS by centrifugation at 1000 rpm for 10 min. After washing PBMNCs were suspended in growth medium [DMEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin-streptomycin (100 U / ml), gentamicin (50 µg/ ml) and 2-mercaptoethanol were cultured at 2×10^5 cells/well in 96 wells round bottom microtiter plates in triplicate wells, three wells without antigen were used as controls. 5 µg/ ml of Con-A was added in triplicate wells, three wells without stimulus were used as controls. Plates were incubated at 37°C under 5% CO₂ for 72 hrs, 4 hrs. before the end of cultivation 50 µl of supernatant was harvested and 50 µl of DMEM complete medium was added, this supernatant was used in NO test. 25 µl per well of MTT dye (5 mg /ml) was added, culture was incubated at 37°C for 4 hrs. After the incubation, 150µl of dimethylsulfoxide (DMSO) was added in each well and mixed it to completely dissolve the purple crystals; plate was incubated at 37°C overnight. The test read at the wavelength 570 nm. For data analysis mean O.D was calculated.

Nitric Oxide (NO) Test

The nitric oxide production by macrophages on stimulation with LPS antigen of *B. melitensis* was assessed using a colorimetric assay for nitrite based on Griess reaction (Stephanie and Glaven, 1989). Nitrite and other reactive

intermediates have been postulated to participate in arginine dependent tumor cytotoxicity by activated macrophages. To perform this assay 50 µl of culture supernatant was collected after 48 hours from the previous experiment (LTT assay) in 96 wells ELISA plate. Nitrite concentration in the supernatant was assayed by a standard Griess reaction. 50 µl of supernatant were added to 100 µl of 1% sulphanilamide, and then 100 µl of 0.1% naphthyl ethylenediamine dihydrochloride was added. Plate was incubated at 37°C for 20 min and absorbance was read at 543 nm.

RESULT AND DISCUSSION

The approximate molecular weight of each fraction collected for LPS antigens was identified by gel filtration chromatography (Table-1; Fig. 1). Stimulation of lymphocytes with fractionated antigen significantly higher compared to control, indicating potential of CMI in response to antigen induced cells. Mitogen (con-A) also gave higher stimulation of lymphocytes than unstimulated but lower than the antigen stimulated lymphocytes. All the LPS fractionated antigen gave higher lymphocytes stimulation than mitogen stimulated lymphocytes. After comparing all the LPS antigen fractions which were fractionated by gel filtration chromatography, it was observed that LPG1 induced maximum lymphocyte proliferation *in-vitro*, followed by LPG2, LPG5, LPG4 and then LPG3 respectively (Fig. 2).

B. melitensis is an intracellular pathogen, hence the role of cell-mediated immunity (CMI) has been proposed in its control, spread and elimination from the host system (Nicoletti and Winter, 1990).

Cellular immune response is a critical part of the host's defence against intracellular bacterial infections. Immunity against *Brucella* species depends on antigen specific T-cell mediated activation of macrophages, which are the major effectors of cell mediated killing of this organism (Oliveira *et al.*, 1998). In addition to the central role of the macrophage in *Brucella* infection, others cells of the immune system are influenced by the interaction between bacteria and host. These cells can counteract the intra-macrophagic development of the bacteria and finally influence the further development of the host defense. The ability of *Brucella* to survive and replicate within host phagocytic and nonphagocytic cells seems to be responsible for the duration of the diseases, which may remain active for years. The protection against *Brucella* infection requires a long lived cellular immune response, depending on the processing of the bacteria by macrophages (Araya *et al.*, 1989; Baldwin and Winter, 1994). This study also showed that *in vitro* proliferation of lymphocytes induced with fractionated LPS antigen was higher in comparison to mitogen induced lymphocytes as well as control. This is an agreement with the previous study (Sinha *et al.*, 2007).

Endothelial cells, macrophages, neutrophils, fibroblasts, and keratinocytes synthesized NO throughout the body. It also serves as an intercellular messenger and as a toxic defense molecule for nonspecific immune responses to infectious pathogens (Sasaki *et al.*, 1998). *In vitro* role of NO for antimicrobial activity has been demonstrated against a broad range of pathogenic microorganisms, including viruses,

bacteria, fungi, and parasites (Groote and Fang, 1995). In this study, there was significantly higher production of nitric oxide by monocyte-derived macrophages on stimulation with different fractionated LPS antigens. The results of this study showed that region LPG1 produce maximum nitrite followed by LPG2, LPG3, LPG5 and LPG4 respectively. It was also observed that insufficient amount of nitric oxide was produced by control and mitogen induced monocytes in comparison to monocytes induced by fractionated lipopolysaccharide antigens (Fig. 3). This *in vitro* study also reported that the production of nitric oxide produced by monocytes induced by fractionated lipopolysaccharide antigens was higher in comparison to control and monocytes induced by mitogen. This study also agrees with the previous study in which synthesis of nitric oxide was observed from activated monocytes induced by fractionated soluble antigen of *B. melitensis* in cell culture (Verma *et al.*, 2016).

CONCLUSION

On the basis of findings of this study, it could be concluded that LPG-1 and LPG-2 fractions of lipopolysaccharide antigens induced proliferation of lymphocytes and also induces monocytes to produce significant amount of NO, therefore it seems that it plays an important role in cell mediated immune response. These antigens may be used for the development of diagnostic kits for the detection of *anti-Brucella* antibodies in an infected host.

Table 1. Table showing peaks, regions and molecular weight range of Lipopolysaccharide (LPS) antigens.

Peak	Region*	Mol. Wt Range (KDa)
I	LPG1	0 – 84
II	LPG2	84 – 78
III	LPG3	78 – 66
IV	LPG4	66 – 45
V	LPG5	45 – 23.5

*LPG: Lipopolysaccharide antigen (LPS) fractionated by gel filtration chromatography

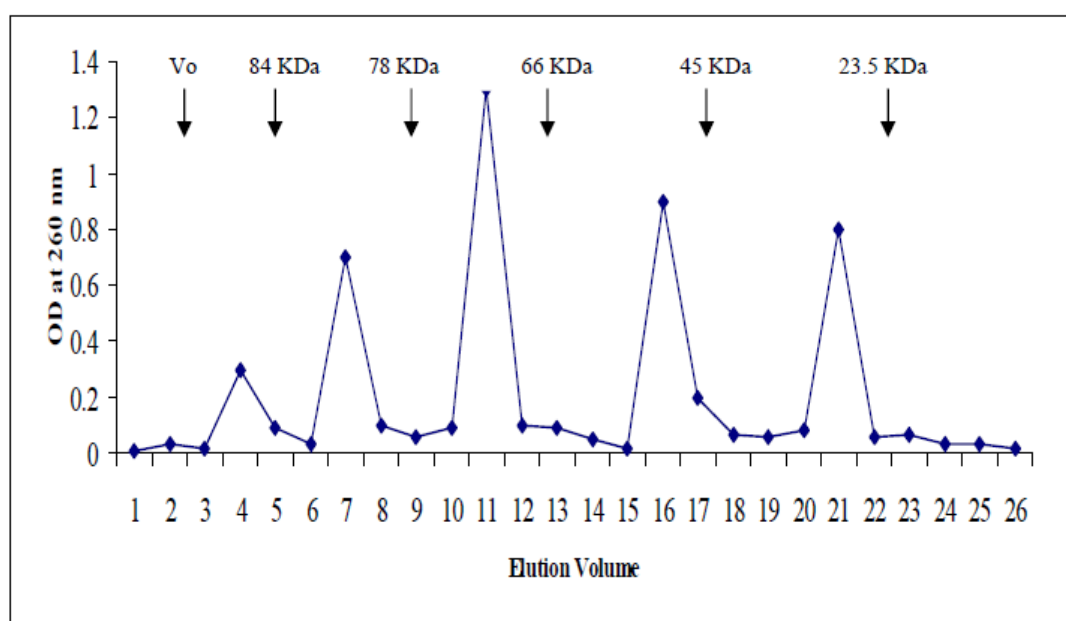


Fig. 1. Sephacryl S-200 of *B. melitensis* LPS antigen.
Area between arrows indicate the fractions pooled under each peak.

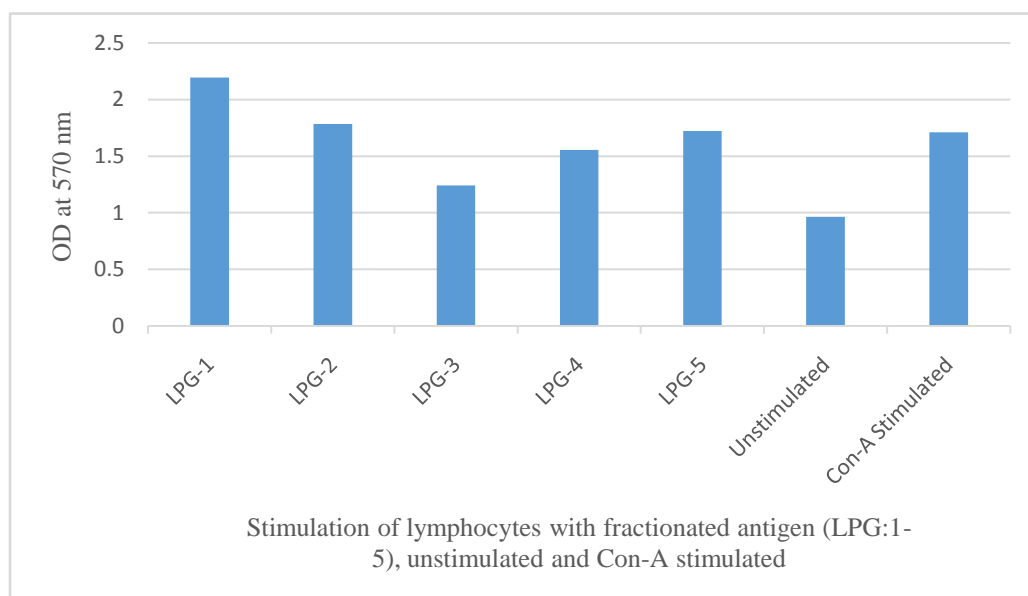


Fig. 2. LTT assay for fractionated Lipopolysaccharide antigens.

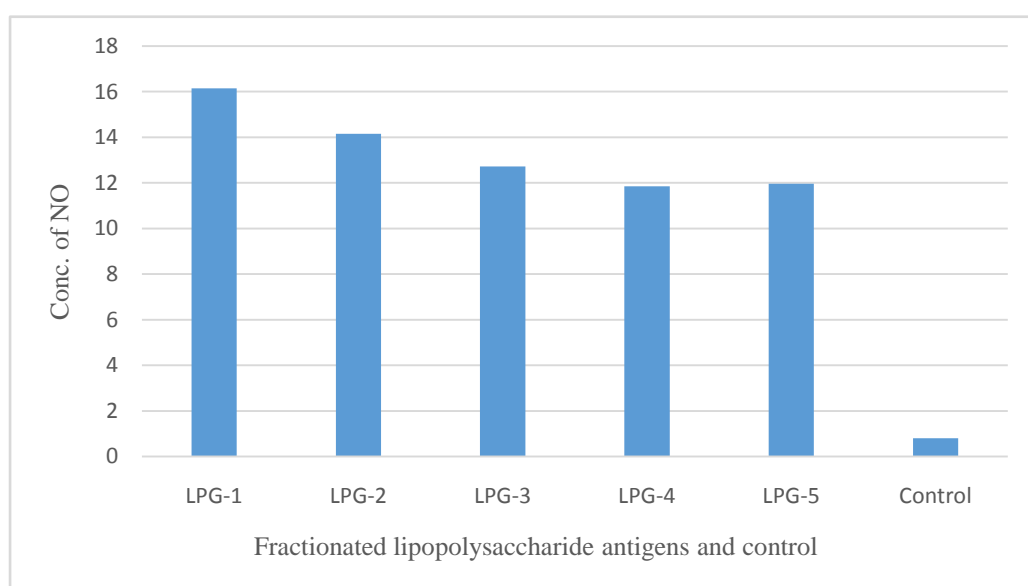


Fig. 3. Nitrite production from monocytes induced with fractioned LPS antigens

ABBREVIATIONS

LPS: lipopolysaccharide; LTT: lymphocyte transformation test; LPG: Lipopolysaccharide antigen (LPS) fractionated by gel filtration chromatography; *B.*: *Brucella*; Con-A: Concanavalin-A; NO: nitric oxide; T_H: T helper cells and T_C: T cytotoxic cells.

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